WANDERING PATHWAYS IN THE REGULATION OF INNATE IMMUNITY AND INFLAMMATION

Alberto Mantovani

Humanitas Clinical and Research Center, via Manzoni 56, 20089 Rozzano (Milan), Italy;
Humanitas University, via Rita Levi Montalcini, 20090 Pieve Emanuele (Milan), Italy;
The William Harvey Research Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ.

Abstract

Tumor-associated macrophages (TAM) have served as a paradigm of cancer-related inflammation. Moreover, investigations on TAM have led to the dissection of macrophage plasticity and polarization and to the discovery and analysis of molecular pathways of innate immunity, in particular cytokines, chemokines and PTX3 as a prototypic fluid phase pattern recognition molecule. Mechanisms of negative regulation are complex and include decoy receptors, receptor antagonists, anti-inflammatory cytokines and the signalling regulator IL-1R8. In this review, topics and open issues in relation to regulation of innate immunity and inflammation and specific issues are discussed: 1) how macrophage and neutrophil plasticity and polarization underlie diverse pathological conditions ranging from autoimmunity to cancer and may pave the way to innovative diagnostic and therapeutic approaches; 2) the key role of decoy receptors and negative regulators (e.g. IL-1R2, ACKR2, IL-1R8) in striking a balance between amplification of immunity and resolution versus uncontrolled inflammation and tissue damage; 3) role of humoral innate immunity, illustrated by PTX3, in resistance against selected microbes, regulation of inflammation and immunity and tissue repair, with implications for diagnostic and therapeutic translation.

1. Encounter with the big eaters: the good, the bad and the never ugly macrophage

I trained as a physician scientist, spending a substantial part of my time in the lab, first at the Institute of General Pathology (Molecular Biology, in pioneering early days), then at the Mario Negri Institute (immunology, Dr. Federico Spreafico). A close encounter with patients at the Istituto Nazionale Tumori in the Department lead by Gianni Bonadonna with Dr. Fossati Bellani had a profound impact on my choice of cancer as a major focus of my research [1]. I met the "big eaters", the macrophages, as part of what was then called the "reticuloendothelial system". A typical assay was injection of india ink and measuring its clearance. I was fascinated by macrophages and therefore applied to work in the laboratory of Robert Evans and Peter Alexander. They had discovered that activated macrophages could kill tumor cells extracellularly [2]. Extracellular killing of tumor cells is a property of classically activated, or M1, macrophages. Siamon Gordon in Oxford later discovered an alternative form of macrophage activation (M2) driven by IL-4 [3]. Many groups including mine have contributed to the characterization of polarized macrophage activation [4-9]. Progress has been made in defining the molecular basis for polarized macrophages activation, including the role of members of the Stat family, NFκB, KLF4, PPARγ, Myc [6, 10]. Epigenetic analysis has added a new dimension to defining the molecular landscape and mechanism involved in macrophage differentiation and polarized activation [11].

The project I was assigned at the Chester Beatty was to investigate the role of macrophages in response to irradiation. In retrospect it was an interesting subject given the role of these cells in tissue repair [12] and the recent evidence linking these cells to response to irradiation [13]. However, at the time the project did not work. At the Mario Negri Institute I had done some work on Adriamycin (now known as Doxorubicin) and therefore I decided to work on that as a side project [14, 15]. I published this paper as "solo" author, because PA (short for Peter Alexander) and Bob said that it was my idea, my work, my paper: what a lesson, and more so now, at a time of diffuse honorary authorship. The role of immunity in the respose to selected chemotherapeutic agents has been revamped by the studies conducted by Laurence Zitvogel and Guido Kroemer on immunogenic cell death [16].

2. Macrophages in cancer: general lessons for immunity and inflammation

After I returned to Milan I focused my attention on macrophages in really malignant mouse tumors, ie a metastatic model and ovarian cancer. The latter provided easy access to ascites, from which it was relatively easy to isolate tumor-associated macrophages (TAM). Much against current views I

found that TAM promoted tumor growth *in vitro* and *in vivo* and again I published a "solo" paper [17, 18]. TAM have served as a paradigm of the connection between inflammation and cancer [19, 20] and illustrated the concept that the microenvironment is a key determinant of tumor progression and a target for therapeutic intervention. Studies on the connection between inflammation and cancer and on immune surveillance [21] propelled a paradigm shift in the vision of the essence of cancer from a cancer cell centric view [22] to one that includes tumor promoting inflammation and tamed adaptive immunity [19, 20, 23, 24]. This change in vision of the essence of neoplasia has been paralleled by the development of immunotherapy strategies including targeting of TAM [13, 25].

TAM have served as a paradigm for the plasticity and polarization of macrophages [4, 7, 12, 26] under a variety of pathophysiological conditions ranging from tissue repair to autoimmunity. Mills and… had used the term M-1 and M-2 referring to macrophages from two strain of mice exhibiting different properties and different propensity to mount a Th1 and a Th2 response, respectively [27]. Unfortunately, the alternative activation by IL-4 discovered by Siamon Gordon [3] was not quoted and discussed. We used M1 and M2 to refer to classically activated, tumoricidal macrophages and M2 to the IL-4 activated macrophages. TAM had M2 properties [4, 7]. M1 and M2 or M2-like are extremes of a spectrum in a universe of activation states [28]. The M1/M2 (and M2-like) nomenclature mirrors Th1/Th2, ILC1/ILC2, type1/type2 immunity etc. On the other hand, macrophage (and T cell or ILC) plasticity defies a simplistic allocation to an M1 or M2 phenotype. I have argued that M1/M2 has has euristic value and that it has served as a useful communication tool within the community of immunologists and with other fields of biomedicine, as shown by its darwinian success [29]. Polarized macrophages have emerged as key players in autoimmunity as illustrated by rheumatoid arthritis [30-32].

3. The small brothers, neutrophils

In the early '80 I went back to molecular biology and decided that I had to get acquainted with the technology which was changing the landscape of research in Immunology and oncology. I went back the National Institutes of Health, which I had visited in 1978 and 1978 (Laboratory of Ronald Herberman) and spent a year in Frederick with Luigi Varesio in the lab of Joost Oppenheim. Capitalizing on that experience, in the context of a study on expression of transcription factors in myelomonocytic cells, we made the unexpected observation that neutrophils express high levels of the c-fos transcription factor [33]. This observation suggested that neutrophils were more than terminally differentiated effector cells and that they could reprogram their function. We went on to

show that bacterial products and selected cytokines, IL-1 in particular, dramatically increase the lifespan of these cells, allowing time for functional reprogramming [34]. Neutrophils have since emerged as active players in induction, regulation and effector phase of diverse forms of immunity [35, 36]. Moreover, evidence suggests that neutrophils can undergo polarization, N1 and N2 in tumors [37], though the signals involved are different from IL-4 . It will be important to assess the role of neutrophils in autoimmunity from the more sophisticated perspective of neutrophil plasticity and their role as sophisticated regulators of immunity.

4. Negative regulators: from cancer to autoimmunity

Once upon a time there was only one cytokine related to inflammation and produced by macrophages, IL-1 [38-40] (see also review by Charles Dinarello in this issue). It was therefore natural get interested in IL-1. In parallel I had the priviledge of starting an invaluable collaboration with Elisabetta Dejana, an expert in endothelial cell biology and a leader in the field. At a time of the "reticuloendothelial system", we engaged in a fruitful and enriching collaboration on the interplay between immunity and vascular endothelium. We discovered that a cytokine "bouille-à-bèsse" caused gene expression dependent reprogramming of endothelial cells and identified the active ingredient as IL-1 [41-43]. The picture which emerged from the studies conducted by us and by others (e.g. [44]) revealed that inflammatory cytokines activated a proinflammatory/prothrombotic program in endothelial cells [45]. Later, given my interest in alternative activation of macrophages, we described how IL-13 affects endothelial cell function in a distinct way [46]. Endothelial cells were once seen as a "sheet of nucleated cellophane" endowed with negative properties, ie not being thrombogenic. Therefore we witnessed a major change in view of the pathophysiology of vascular biology and of its interplay with the immune system.

Siamon Gordon described an alternative, M2 form of macrophage activation [3]. The context of an analysis of the interplay of IL-4 with components of the IL-1 system, we identified the type 2 IL-1 receptor (IL-1R2) as a decoy for IL-1 upregulated by IL-4 and glucocorticoid hormones [40, 47]. The classic definition of "receptor" involves ligand recognition and signalling. The discovery of a decoy receptor was without precedent in biology. Decoy receptors have since emerged as a general strategy to tune the action of cytokines, chemokines and growth factors. Decoy receptors have been identified in Drosophila and therefore they represent an evolutionary ancient strategy of regulation.

After the discovery of IL-1R2 as a decoy, negative regulation has become a recurrent theme in my laboratory. We cloned one of the isoforms of the IL-1 receptor antagonists [48] and, stemming from our interest in IL-1, discovered that the MyD88 adaptor was downstream of at the time only human Toll, now TLR4 [49]. We have since been engaged in the search and characterization of atypical receptors which we hypothesized, based on structural considerations, should not behave as signalling or conventionally signalling receptors. Massimo Locati identified D6, now ACKR2 [50] as candidate decoy for inflammatory CC chemokines based on structure. His prediction was vindicated by data [51, 52]. ACKR2 is a decoy and scavenger for inflammatory CC chemokines essential for the regulation of inflammation, resolution of inflammation and cancer-related inflammation) [52, 53].

In the 1990s we first molecularly cloned and deposited the sequence of a fringe member of the IL-1 receptor family, TIR8 [54, 55]. Since it was the 8th molecule with a TIR domain we called it TIR8. Independently it was identified as SIGIRR [56] and we now refer to this receptor as IL-1R8. IL-1R8 has unique structural features relative to other members of the ILR family: a single Ig domain, two substitutions in the TIR domain incompatible with conventional signalling, a long cytoplasmic tail [40]. We hypothesized that IL-1R8 be a negative regulator. Evidence suggests that IL-1R8 is recruited at signalling receptor complexes of members of the IL-1 and TLR receptor families, interferes with formation of a "MyDosome" and dampens signalling [40, 56, 57]. Moreover, recently, it was observed that IL-1R8 is part of the receptor complex that recognizes IL-37, an antiinflammatory cytokine [58]. IL-1R8 deficient mice exhibit uncontrolled inflammation in response to infection, inflammatory signals and autoimmunity [40, 59]. Therefore work conducted with negative regulatos (e.g. IL-1R2, ACKR2, IL-1R8) characterized by different structure and ligands has highlighted a recurrent theme illustrated in Fig. 1. Negative regulators are essential to strike a balance between amplification of innate immunity, and activation of adaptive responses, versus uncontrolled, non-resolving host damaging inflammation. Uncontrolled non-resolving inflammation drives tissue destruction, cancer and autoimmunity.

Studies on IL-1R8 recently took an unexpected turn. We found that IL-1R8 is highly expressed in human and murine NK cells [60]. Here IL-1R8 serves as a negative regulator of the response to IL-18 which drives differentiation and activation of NK cells. Genetic inactivation of IL-1R8 unleashes NK cell-mediated resistance against liver carcinogenesis and hematogenous metastasis in liver and lung. These organs are characterized by abundant presence of NK cells. Thus, the organ immunological context is a key determinant of the efficacy against cancer of different components of the immune system. The checkpoint function of IL-1R8 and its role in taming NK cell-mediated antitumor and antiviral resistance call for exploration of its therapeutic

value. In previous studies, IL-1R8 deficient mice were more susceptible to autoimmunity [40, 59]. These recent results [60] call for a reappraisal of the role of IL-1R8 in lymphoid cells in the context of autoimmunity.

5. The power of attraction: chemokines and more

The finding indicating that macrophages promoted tumor growth raised the hypothesis that tumor cells attracted macrophages to fuel tumor growth and metastasis. We identified a chemoattractant produced by tumor cells active on monocytes and not on neutrophils, associated with TAM infiltration [61]. Classic chemoattractants known at the time, formylpeptides and Complement components, were active on both monocytes and neutrophils. We called the active principle tumor-derived chemotactic factor (TDCF) in spite of the fact that we knew that it could be produced also by normal cells, a nomenclature mistake on my side [61]. TDCF was one of the pathways which lead to the identification of the chemokine MCP-1/CCL2 [62] and was our port of entry to the chemokine universe. Our contributions to the field included the characterization of chemokines attracting immature and mature dendritic cells [63], the characterization of CCL22 [64], the discovery that polarized T cells express distinct repertoirs of chemokine receptors [65]. Our chemokine efforts eventually merged with negative regulators and decoy receptors as discussed above.

While continuing to dissect the connection between known chemoattractants and cancer [66, 67], I am now back to characterize candidate novel chemoattractants stemming from my roots, macrophages in cancer [68].

6. Humoral innate immunity

The innate immune system includes a cellular and a humoral arm [69, 70]. The cellular arm of innate immunity is based on pattern recognition receptors such as TLRs, which have represented a quantum leap in our understanding of sensing of microbes and tissue damage by innate immunity cells. With the exception of Complement [71, 72] humoral innate immunity is frequently represented as a collection of weird molecules (e.g. ficolins; mannose binding lectin; C reactive protein). We stumbled in humoral innate immunity in the context of our interest in IL-1. Going fishing for IL-1 inducible genes in collaboration with the laboratory of Elisabetta Dejana, we cloned PTX3, a humoral fluid phase pattern recognition molecule [66, 73, 74]. We decided to focus on

PTX3 because it looked like a distant relative of the pentraxin C reactive protein (CRP), an acute phase protein and diagnostic marker in inflammatory diseases whose actual function cannot be assessed using rigorous genetic tools because of lack of evolutionary conservation [69, 70, 75] . Our hypothesis was that the PTX3 would give us an insight into the logic of humoral innate immunity. PTX3 has antibody-like properties: it recognizes microbial components, has opsonic activity, activates and regulates the Complement cascade [69, 70, 75]. A discussion of the multifaceted functions and structure of PTX3 is beyond the scope of this essay. In terms of translation, under many inflammatory conditions PTX3 is an earlier biomarker, better related to prognosis than CRP [75]. PTX3 genetic polymorphisms are associated to susceptibility to selected infectious agents, *Aspergillus fumigatus* in particular [76-78]. The role of PTX3 in autoimmune conditions has not been extensively investigated [75]. For instance, interestingly, in small vessel vasculitis, PTX3 levels emerged as a correlate of disease severity, possibly involved in pathogenesis [79].

7. Perspective and a challenge

The field of innate immunity has moved from being tangential to mainstream immunology to taking central stage in terms of fundamental mechanisms and pathogenesis of disease. I have had the privilege of seeing this profound change of perspective and of being part of it. I focused my efforts on fundamental mechanisms of immunity and inflammation and cancer. Here, the dissection of fundamental mechanisms and a move from a cancer-cell centric view of cancer to one which includes inflammation and immunity [19, 24] have paved the way to therapeutic exploitation [80]. The development of immunotherapy is the initial fulfilment of a dream of generation of physicians and scientists since the birth of modern medicine. Again I had the privilege to witness and be part of this adventure. A recurrent theme in cancer and autoimmunity is the failure of resolution of inflammation. Some of the molecules which I discussed here are part of the complex network which underlies resolution [81]. Translating dissection of resolution into diagnostic and therapeutic tools remains a formidable challenge with broad implications in human disease.

Acknowledgments

Financial support by AIRC, ERC, CARIPLO, Italian Ministry of Health is gratefully acknowledged. This paper is dedicated to the PhD students, postdoctoral fellows, medical students and young MDs, who worked in my laboratory over many years and brought dedication and

creativity. A special word of gratitude to my outstanding technicians, all represented by “Beppe” Peri who passed. All of them have been Maestros for me.

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Legend to Figure 1.

The role of negative regulators in balancing immunity and inflammation-driven tissue damage and in guiding resolution.